

HETEROPOLYMERS: A NEW CLASS OF THERAPEUTICS FOR TREATING LETHAL BACTERIAL AND VIRAL INFECTIONS

Nehal Mohamed¹, Steven M. Jones¹, Juan Li¹, Claudia Ferreira¹, Xun Chen¹, Barry Kreiswirth²,
Leslie S. Casey¹, George L. Spitalny¹, Steven E. Pincus¹
Elusys Therapeutics, Inc., Pine Brook, NJ ¹, Public Health Research Institute, Newark, NJ²

ABSTRACT

Heteropolymers (HPs) contain a MAb directed against the Complement Receptor type 1 (CR1) on primate RBCs, chemically cross-linked to MAbs that recognize pathogens. Upon administration of HPs pathogens are bound to the HP and immobilized on RBCs, which move through the circulation to the liver and spleen where the CR1-HP-pathogen complex is phagocytosed and destroyed by tissue macrophages. A unique feature of HPs is that the anti-pathogen MAb can be directed to a non-neutralizing epitope on the target organism. HPs are efficacious in treating lethal bacterial and viral infections and can be used as prophylactic for biowarfare agents.

INTRODUCTION

The U.S. public health system and primary healthcare providers must be prepared to address biological pathogens that are rarely seen in the U.S. High priority agents (CDC/NIAID Category A Bioterrorism Agents) include organisms that pose a risk to national security because they (1) can be easily disseminated or transmitted from person to person; (2) result in high mortality rates and have the potential for major public health impact; (3) might cause public panic and social disruption and (4) require special action for public health preparedness. Among these agents are bacterial and viral diseases (see Table 1).

TABLE 1. CDC/NIAID Category A Bioterrorism Agents.

| <u>Bacterial</u> | <u>Viruses</u> |
|---|---|
| <ul style="list-style-type: none">• Anthrax• Yersinia pestis (Plague)• Francisella tularensis (Tularemia)• Clostridium botulinum | <ul style="list-style-type: none">• Poxviruses (Smallpox)• Viral hemorrhagic fevers<ul style="list-style-type: none">Filoviruses (Ebola, Marburg)Arenaviruses (Lassa, Machupo)Bunyaviruses (Hantavirus, Rift Valley Fever)Flaviviruses (Dengue) |

| Report Documentation Page | | | | Form Approved OMB No. 0704-0188 | |
|--|------------------------------------|-------------------------------------|--|---|------------------------------------|
| Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. | | | | | |
| 1. REPORT DATE 17 NOV 2004 | | 2. REPORT TYPE N/A | | 3. DATES COVERED - | |
| 4. TITLE AND SUBTITLE Heteropolymers: A New Class Of Therapeutics For Treating Lethal Bacterial And Viral Infections | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Elusys Therapeutics, Inc., Pine Brook, NJ | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004 . , The original document contains color images. | | | | | |
| 14. ABSTRACT | | | | | |
| 15. SUBJECT TERMS | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT UU | 18. NUMBER OF PAGES 13 | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT unclassified | b. ABSTRACT unclassified | c. THIS PAGE unclassified | | | |

There clearly is a need to develop vaccines and therapeutics for these category A agents to protect U.S. government workers and the public in the event of a bioterrorist attack. HP technology provides a new tool for the development of prophylactic and therapeutic drugs against category A agents. The HP technology is based upon the natural mechanism for clearance of blood-borne antigens of the primate immune system, immune adherence (IA) (Figure 1A)¹. When antibodies of the right subclass bind their antigen, which can be a protein on the surface of a virus or bacteria, they form an immune complex (IC) that activates the complement system. During activation, one of the complement proteins, C3b, attaches to the Fc region of the antibody followed by binding of the IC to complement receptor type 1 (CR1) on red blood cells (RBCs)². About 90% of CR1 in humans is found on RBCs, although CR1 is expressed on some nucleated cells as well.³ RBC-CR1-bound ICs are transported to the fixed macrophages, primarily the liver, where cleavage of CR1 by a membrane associated protease releases the IC, which is finally internalized and destroyed by the fixed tissue macrophages.^{2,4} Because hCR1 is a privileged site on RBCs that has evolved for transport of IC's, after cleavage of CR1, the RBCs return to the circulation undamaged and continue to perform their normal physiological function.⁴

HP technology operates in a more efficient manner than IA to remove antigen (human pathogens) from the circulation. HPs are composed of two monoclonal antibodies: one specific for the CR1 molecule and the second specific for the target pathogen. HPs have been shown experimentally to bind a wide variety of target antigens in the bloodstream to erythrocyte CR1 without the requirement to activate complement, and to transfer the antigens to fixed tissue macrophages via FcγR.² HPs supply the pathogen specific antibody, thus providing “instant immunity” without the need for the development of an immune response to generate antibodies that recognize the pathogen. By entirely bypassing the need to activate the complement cascade for binding to CR1 on RBCs, HPs significantly increase the efficiency of target antigen clearance to the surface of the erythrocyte, and dramatically improve upon the efficiency with which the natural IA mechanism effects destruction of blood-borne pathogens by fixed tissue macrophages.

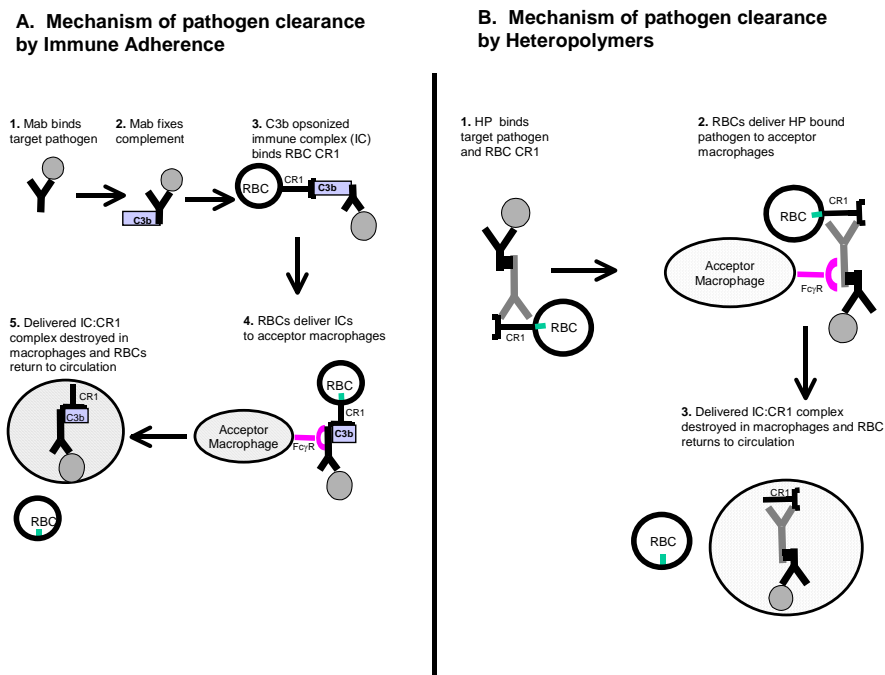


FIGURE 1. Mechanism of pathogen clearance by Immune Adherence and Heteropolymers.

HPs provide several advantages over conventional treatments. First, the MAb that recognizes the pathogen does not need to neutralize the pathogen because the HP ultimately transports the pathogen to macrophages where the pathogen is destroyed. In theory a single HP molecule is all that is required to clear one pathogen by binding it to the red cell, unlike neutralizing antibodies in which many molecules are required to surround the pathogen in order to inactivate it. Second, HP administration confers instant immunity since the pathogen specific antibody is part of the HP, allowing clearance of the pathogen in the absence of host immune response or in situations where the patient is immunocompromised. Finally, HP mediated clearance of pathogens is complement independent and can function in situations where there is complement deficiency.

Studies conducted in well over 100 non-human primates (NHPs), HPs have proven safe and have been shown to bind viral and bacterial pathogens to RBCs and thus mediate their clearance from the circulation (summarized in Table 2).⁵ However, due to cost and ethical considerations, efficacy studies in NHPs have been limited to small numbers of animals and for limited durations.

TABLE 2. Bacterial and Viral targets cleared in vivo in NHP by HP

| Virus or Bacteria | Clearance of target from circulation | Protection from challenge |
|------------------------|--------------------------------------|---------------------------|
| Bacteriophage ΦX174 | + | NA ¹ |
| Dengue Virus Type 2 | + | ND ² |
| E. coli 0157 | + | ND |
| Pseudomonas aeruginosa | + | 24h post-challenge |

¹Not applicable since ΦX174 is not pathogenic in primates

²Not determined in the study

To expedite efficacy testing of HPs against lethal bacterial or viral challenges we developed challenge models in a transgenic mouse (TgN hCR1) that expresses human CR1 on the surface of their RBCs.⁶ While mice express murine CR1, this molecule is not recognized by MAbs that recognize human CR1, and mouse CR1 is predominantly expressed on platelets whereas human CR1 is expressed on RBCs. The properties of hCR1 on RBCs from the TgN mice were shown to be very similar to the properties of hCR1 on human RBC (summarized in Table 2). To carry out pathogen challenge studies a colony of specific pathogen free TgN hCR1 mice has been established.

TABLE 3. Properties of hCR1 expressed on red blood cells from human or TgN hCR1 mice.

| Property | Human | TgN Mice |
|--|--|-------------------------------------|
| Molecular Weight | 200kDa/190 kDa | 190 kDa |
| Approx. # of CR1 molecules/ RBC | 100-1000 | 2000 |
| Clustered Appearance | Yes | Yes |
| Binding of Immune Complexes in vitro | Yes | Yes |
| Cells expressed on other than RBC | Lymphocytes, follicular dendritic cells, neutrophils, glomerular podocytes | Platelets, CD45 positive population |
| Binding of HP in vivo/in vitro | Yes | Yes |
| Clearance of target antigen in vivo/in vitro | Yes | Yes |

HP MEDIATED PROTECTION FROM LETHAL *STAPHYLOCOCCUS AUREUS* CHALLENGE

To determine if an HP would be an efficacious prophylactic against a bacterial challenge we developed a lethal challenge *Staphylococcus aureus* (SA) model in the TgN hCR1 mice. An advantage of staph as a model pathogen is that relevant animal studies can be conducted at Biosafety level 2, as opposed to BSL-3 or -4 for most biothreat agents. Experiments were performed using SA strain 13301, a

penicillin resistant strain (ATCC, VA). The dose required for 50% lethality in hCR1 mice (LD₅₀) by intravenous (iv) injection was determined to be 10⁶ cfu/mouse (data not shown). In addition, we have used a clinical isolate, the methicillin resistant SA (MRSA) strain, MW2.⁷ The LD₅₀ for this strain was determined to be 2x10⁶ cfu /mouse.

To obtain efficacy data for HP treatment we generated HPs that consisted of a MAb (Spa 27) that recognizes protein A, a protein expressed on the surface of SA, cross-linked to a murine MAb that recognizes human CR1. Protein A functions as an anti-phagocytic protein that SA uses to avoid the immune system by binding IgGs via the Fc portion.⁷

To test HP for efficacy, mice (10 per group) were injected iv with PBS, MAb (40 µg per mouse) or HP (16 µg, 8 µg, 4 µg, 2 µg or 1 µg per mouse). One hour later the animals were challenged with 10x LD₅₀ of MRSA strain MW2 and monitored for time to death (TTD) for 28 days. A Kaplan Meier plot of the survival data is shown in Figure 2. All PBS controls and MAb treated animals died by day 3. However, at an HP dose of 16, 8 or 4µg, all the mice were protected from death. At an HP dose of 2 µg, 9 of 10 mice were still alive at day 28, and at 1µg 7 of 10 mice survived through day 28.

In this study a MAb that recognizes protein A provided protection when incorporated into an HP, but did not neutralize the bacteria and confer protection when administered alone at an ~80-fold greater concentration (40µg MAb versus 1 µg HP, assuming HP is a dimer then 1 µg HP is equivalent to 0.5 µg Spa27 MAb). These data demonstrate that an HP generated with a MAb that can bind, but does not neutralize or protect mice from a lethal SA challenge can be used in HP to develop an effective therapeutic.

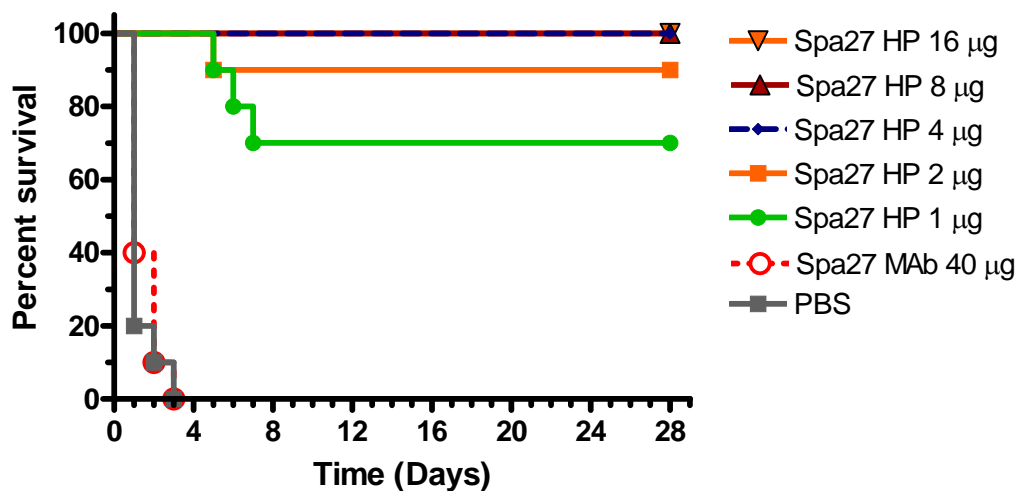


FIGURE 2: Efficacy of HP vs MAb in protection of TgN mice challenged with a lethal dose of MRSA MW2.

GENERATION OF PROTECTIVE IMMUNITY IN HP PROTECTED MICE

It has been postulated hCR1 may function to present pathogens to the immune system.⁸ Therefore, HP mediated protection may lead to the generation of an immune response that can provide protection to a secondary challenge. To test this hypothesis, we asked whether mice protected from a lethal SA challenge by HPs were protected from secondary challenge with the same or a different strain of SA. Mice (10/group) were administered HP (20 µg), MAb (10 µg) or PBS followed by challenge with 10xLD₅₀ of SA strains MW2. As expected, all mice in the MAb and PBS group died by day 5 (data not shown) while all of the mice in the HP group survived out to day 28 (data not shown). On day 28, HP treated survivors were divided into new challenge groups (5/group) and were re-challenged with either

SA 13301 or MW2 (See insert in Figure 3). All of the re-challenged mice survived to day 28, whereas age matched controls challenged with either 13301 or MW2 died by day 3.

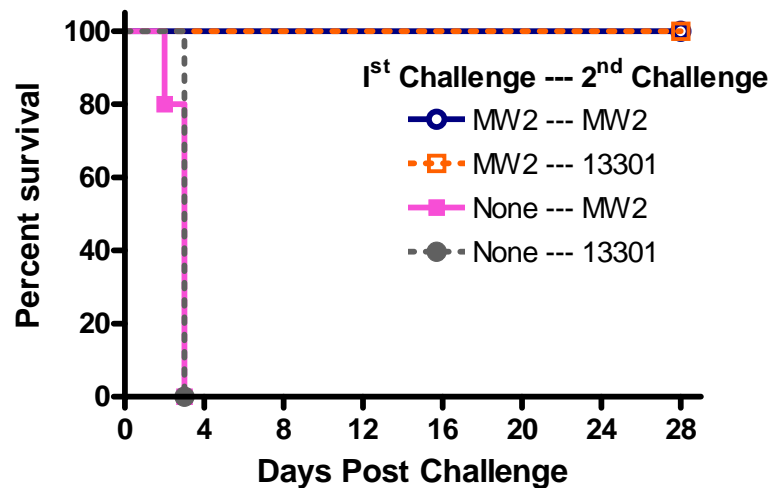


FIGURE 3: Re-challenge of mice protected by HPs from primary SA challenge.

These data demonstrate that HP treated mice that survive a primary SA challenge are protected from re-challenge with the same or a different SA strain.

MICE PROTECTED FROM *S. AUREUS* SURVIVE RE-CHALLENGE BY *S. EPIDERMIDIS*

Since mice that are protected from a primary *S. aureus* challenge by HP treatment are protected against re-challenge with a different SA strain, we wanted to determine whether they would be protected against a challenge with *S. epidermidis*. We established the LD₅₀ for iv administration of *S. epidermidis* strain 10683 in the hCR1 TgN mice (1 LD₅₀=10⁷cfu/mouse). To determine whether HP-SA protected mice could resist a *S. epidermidis* challenge, mice 28 days post HP administration/*S. aureus* challenge or naïve animals that had not experienced any prior challenge were challenged (10 mice/group) with 10 LD₅₀ *S. epidermidis*. The results in Figure 4 show that while all naïve mice died by day 4, all mice that survived *S. aureus* challenge by HP treatment survived *S. epidermidis* challenge.

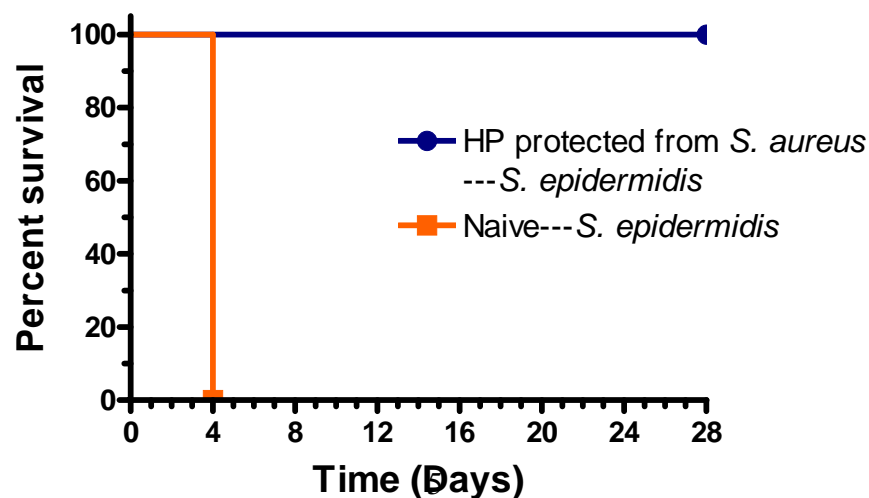


FIGURE 4: Mice protected from *S. aureus* challenge by HP survive re-challenge with *S. epidermidis*.

These data demonstrate that HP mediated protection from a primary SA challenge generates an immune response that is protective not only against a different SA strain but also against *S. epidermidis*.

ANTIBODIES TO SA AND *S. EPIDERMIDIS* IN RE-CHALLENGED MICE

The observed protection of HP treated mice upon re-challenge 28 days later was not due to residual HP on RBCs as pharmacokinetic studies showed that there was no HP remaining on the RBCs after day 14 (data not shown). Thus, the survival of HP treated mice following a re-challenge with SA

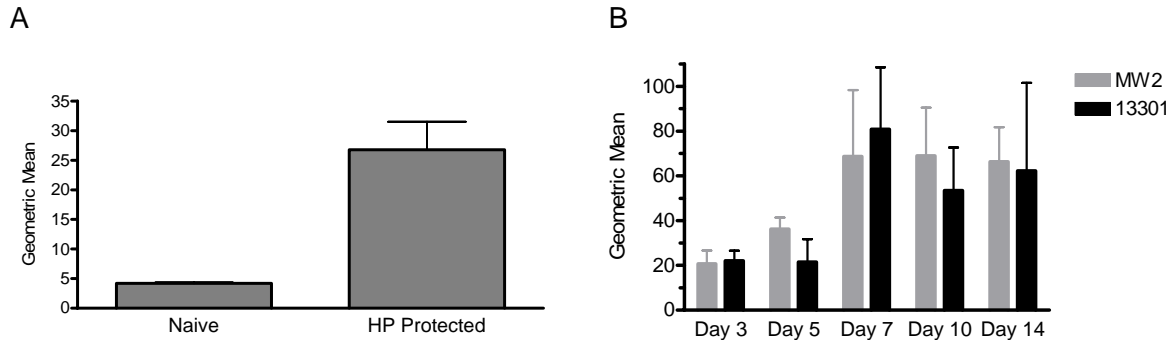


FIGURE 5: Antibody response in HP treated mice. **A.** Antibody responses in Non-treated (Naive) and HP protected mice (28 day post primary challenge). **B.** Antibody response HP protected animals after re-challenge with SA strains MW2 and 13301.

suggests that mice generated a protective immune response. To identify an antibody response in the HP treated survivors sera from naïve, HP protected (28 day postprimary challenge) and re-challenged mice were analyzed for the presence of antibodies that recognized SA. Mice were bled and serum samples were prepared over the course of 14 days post re-challenge. The serum samples were tested for binding to SA using a flow cytometry based assay. Briefly, 5×10^6 cfu of SA strain 13301 was incubated with normal human serum for 1 hour at 37°C to block the protein A binding sites, followed by incubation with mouse serum samples for 1 hour at 37°C. The SA samples were washed and the bound mouse antibodies detected with FITC labeled goat-anti-mouse F(ab')₂. The cells were washed after incubation for 30 minutes at 37°C and analyzed using flow cytometry. As shown in figure 5A, 28 days post primary challenge mice had significant antibody titers to SA (approximately five-fold greater signal compared to naïve sera). Upon re-challenge with SA the antibody titer increased significantly by day 7 post re-challenge (approx. 15-fold signal compared to naïve sera, Figure 5B). It should also be noted that mice that were re-challenged with either SA strain 13301 or MW2 elicited an equivalent response even though the first challenge was with SA strain MW2.

HP protected mice develop an immune response that protects them against re-challenge with a with *S. epidermidis*. It would therefore be predicted that HP protected animals would have antibodies that recognize *S. epidermidis*.

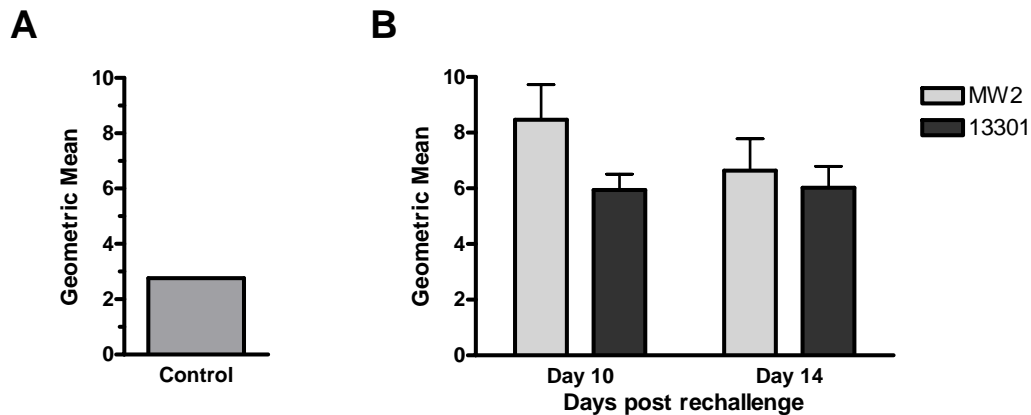


FIGURE 6: Mice protected from *S. aureus* challenge by HP have antibody responses to *S. epidermidis*.

To test this idea anti-*S. epidermidis* antibody titers in sera from mice that survived a secondary challenge with SA strains MW2 and 13301 were analyzed by indirect immuno-fluorescence using flow cytometry. Compared to the fluorescence seen with naïve sera (Figure 6 Panel A) sera from mice 10 or 14 days post SA re-challenge cross-reacted with *S. epidermidis* (Figure 6 panel B, three-fold increase in geometric mean fluorescence). These results clearly demonstrate that the antibody response in HP protected animals is not only broadly reactive and protective against various SA isolates but is also reactive and protective against other staphylococci species.

ANTIBODIES ARE SUFFICIENT TO PROVIDE PROTECTION FROM *S. AUREUS* IN MICE

The presence of antibodies to *S. aureus* in HP-protected mice raises the question, “Are there antibodies that neutralize the bacteria and thus responsible for providing protection”? To address this question we performed a passive immune transfer experiment. To generate a pool of immune sera, HP-protected mice were re-challenged on day 28 with SA and bled on day 14-post secondary challenge and sera prepared. As a control, naïve mice were bled and sera prepared. The equivalent of ~0.5 mouse worth of serum was injected IP into naïve mice (8 mice/group) and these mice were challenged 1hr later with SA strain MW2. A control group of mice received PBS 1hr before challenge. All mice that received PBS or naïve sera died on day 1 post-challenge (Figure 7). There was a statistically significant increase in time to death in mice that received sera from HP-protected mice and 2 of 8 of mice survived for 28 days post-challenge. These results demonstrate that antibodies generated in HP-protected mice are sufficient to provide protection from SA challenge and may be responsible for some or all of the protection in HP protected mice that survive a re-challenge.

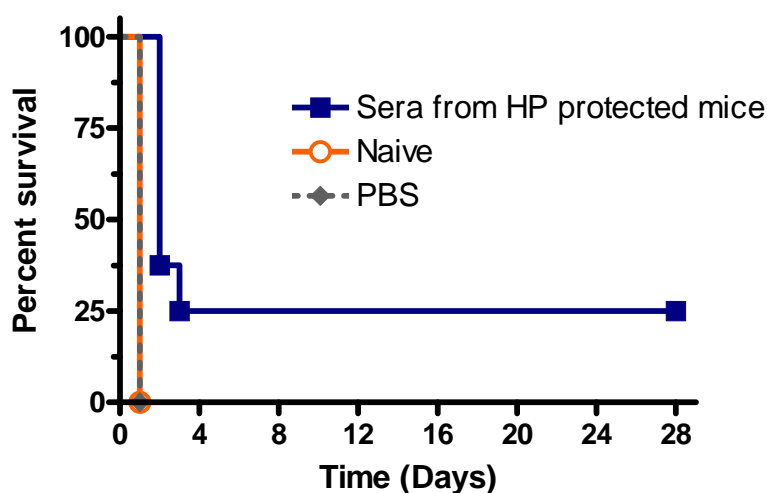


FIGURE 7: Protection from *S. aureus* challenge by transfer of sera from HP-protected mice.

SUMMARY OF RESULTS FROM HP MEDIATED PROTECTION AGAINST SA CHALLENGE

The data presented so far have demonstrated the utility of HPs as a prophylactic treatment for a bacterial infection. Several significant findings have been discussed: (1) a non-neutralizing SA antibody which failed to protect against SA challenge is effective if conjugated to create an HP, (2) HP protected animals develop an immune response that protects against re-challenge with other staphylococci, (3) the immune response in HP protected animals results in antibodies that recognize other staphylococci and (4) these antibodies can confer protection against SA challenge.

HP MEDIATED PROTECTION FROM LETHAL WEST NILE VIRUS OR VACCINIA VIRUS CHALLENGE

As reviewed in Table 1, there are a number of viruses that are classified as high priority (CDC/NIAID Category A) because of their potential as bioterrorism agents. To determine if an HP therapeutic would be efficacious in treating viral infections, models for two important viral pathogens West Nile Virus (a flavivirus) and vaccinia virus (a poxvirus) were developed in the TgN hCR1 mice..

We developed a model of West Nile Virus infection (WNV) in the TgN hCR1 mice using the NY99 WNV isolate. Initial experiments were conducted to establish a WNV challenge dose in TgN hCR1 mice. These experiments established that a challenge dose of 200 PFU (30-50 LD₅₀ reproducibly resulted in \geq 90% lethality and that males and females were equally sensitive (data not shown).

To obtain efficacy data for HP treatment a HP was prepared that consisted of a murine MAb (4G2) that recognizes an epitope on the envelope protein of WNV as well as other members of the flavivirus family including Dengue, cross-linked to murine MAb 7G9 that recognizes hCR1. The 4G2 MAb has previously been shown to neutralize some flaviviruses but has limited neutralizing activity against WNV (Dr. Peter Mason; personal communication).⁹ To determine the efficacy of the 4G2-7G9 HP, mice (16 per group) were injected ip with PBS, HP (10 μ g or 1 μ g per mouse) or MAb (5 μ g or 0.5 μ g per mouse, equivalent to the dose present in the HP group assuming the HP is a dimer). Twenty-four hours later the animals were challenged ip with 20 PFU WNV and monitored for time to death (TTD) for 14 days. Kaplan Meier plots of the survival data are shown in Figure 8.

Significant protection from a WNV challenge was observed in mice treated with either 10 μ g HP (9/16 survivors) or 1.0 μ g (6/16 survivors) (Figure 8), whereas no significant protection was observed in mice treated with MAb alone (3/16 survivors at 5 μ g or 0/16 survivors at 0.5 μ g) compared to PBS controls (1/16 surviving). Future studies will determine the protection achieved by a higher dose of HP and will also examine whether the 4G2 HP can provide protection against Dengue virus challenge.

These results demonstrate that HPs can provide protection against a lethal WNV challenge and the WNV antibody in the HP does not have to be neutralizing.

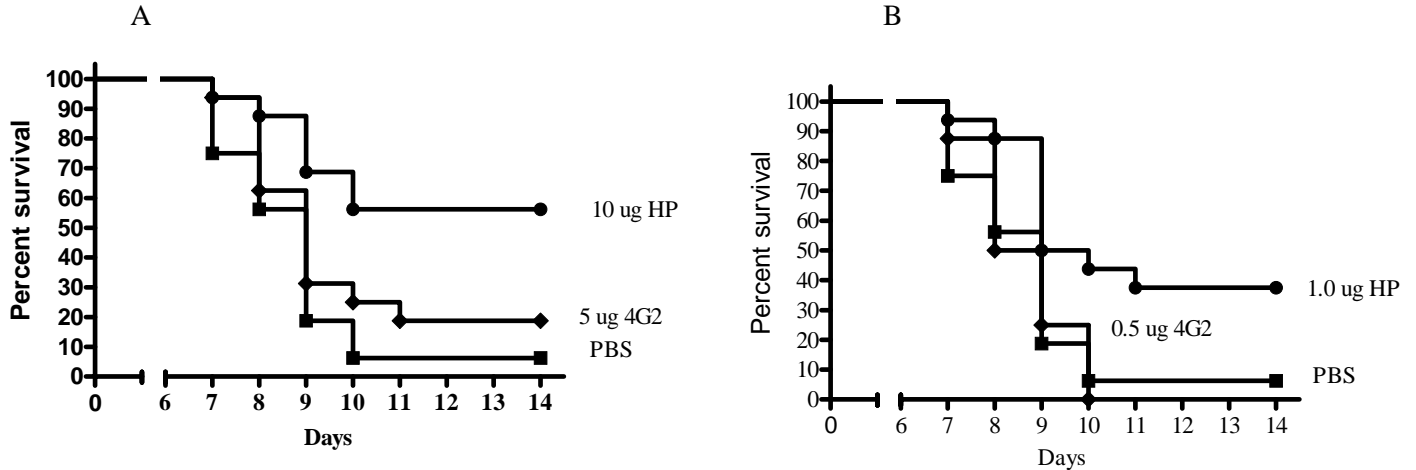


FIGURE 8: Efficacy of HP versus MAb in protection of mice challenged with a lethal dose of WNV. A. Comparison of protection afforded by 10 μ g HP or the equivalent amount (5 μ g) of MAb. B. Comparison of protection afforded by 1.0 μ g HP or the equivalent amount (0.5 μ g) of MAb.

To determine whether HPs would be applicable to multiple viruses, a vaccinia challenge model was developed in the TgN hCR1 mice using the WR strain of vaccinia. Experiments established that the LD₅₀ for WR administered IP to TgN hCR1 mice was 6×10^6 PFU.

Multiple forms of viral particles are produced during the assembly of vaccinia virus. Intracellular mature virions (IMV) are wrapped by a single lipid membrane,¹⁰ can be released from disrupted cells and are a fully infectious form of the virus. IMV particles are physically robust and are well suited for dissemination between hosts. IMV can be wrapped in two additional membranes and move to the cell periphery where the outermost membrane fuses with the cell plasma membrane forming cell-associated enveloped virions (CEV).¹¹ CEV is released from cells as extra cellular enveloped virions (EEV), which are infectious and play a role in long-range spread of the virus¹² and cell-to-cell spread.¹³

HP's were prepared by cross-linking a MAb that recognizes hCR1(7G9) to a non-neutralizing MAb specific for IMV (IMV-HP) or a non-neutralizing MAb specific for EEV (EEV-HP). The specificity of each HP for IMV or EEV forms of virus was confirmed *in vitro*. Mice were injected IV with 95 μ g IMV-HP, 70 μ g EEV-HP or PBS and challenged 1 hr later by IP administration of ~ 5 LD₅₀ vaccinia virus and monitored for TTD for 21 days. A Kaplan Meier plot of the survival data is shown in Figure 9.

Mice that received IMV-HP were significantly protected from vaccinia challenge with 3/6 surviving ($P=0.0023$) compared to mice that received either PBS (0/6) or the equivalent dose of EEV-HP MAb (0/6, $P=0.02$). Future studies will determine the protection achieved by combining the IMV and EEV-HPs and comparing the results with that seen upon individual or combination administration of the Mabs.

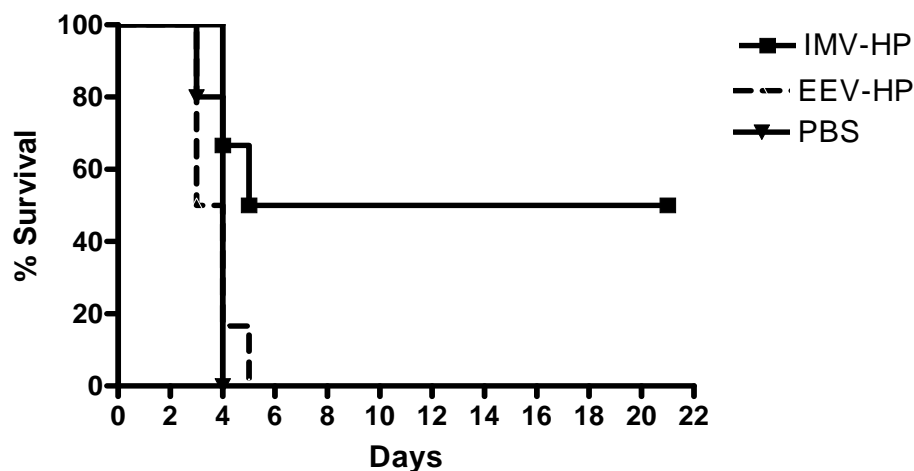


FIGURE 9: Efficacy of HP's in protection of mice challenged with a lethal dose of vaccinia virus.

These data combined with the WNV data indicate that HPs can be developed as effective therapeutics for treatment of viral infections.

DISCUSSION

The results presented above demonstrate that heteropolymers (HPs) are effective prophylactics against bacterial and viral pathogens and support the further development of HP therapeutics against category A bioterrorism agents. Furthermore these results provide evidence that non-neutralizing antibodies are sufficient to generate efficacious HPs. This provides the opportunity to develop broad acting HPs that can clear multiple pathogens by generating HPs from non-neutralizing antibodies that recognize shared common epitopes on a family of bacteria or virus. For example, these studies have utilized MAb 4G2, a flavivirus cross-reactive MAb that when conjugated as an HP provides protection against WNV, and should also provide protection against the 4 serotypes of dengue. Future studies are planned to test this possibility.

Preliminary data indicate that an HP administered 6h after SA challenge resulted in significant protection (data not shown). Based upon the number of RBC/ml (5×10^9) and the range of CR1 molecules on human RBC (100-1000) one can calculate that up to 2.5×10^{12} pathogens can be bound to human RBC per ml of blood if an HP can bind to two CR1 molecules and simultaneously bind one pathogen. Assuming that HP is a dimer, this would represent approximately a $1.3 \mu\text{g/ml}$ dose. Since the capacity for clearing pathogens clearly exceeds the levels of virus or bacteria ($< 10^{10}$ particles/ml) that will be present during an active infection, the therapeutic dose of an HP is predicted to be $\leq 13\text{ng/ml}$, far below the doses that are administered for therapeutic antibodies.

The ability to provide prolonged prophylaxis by HPs is demonstrated in the WNV challenge, the 4G2 HP was able to provide protection when administered at 1 and $10 \mu\text{g/mouse}$ ip 24h prior to challenge (Figure 8). Pharmacokinetic studies have demonstrated that the half-life of the 4G2 HP on the RBCs is ~62h (2.5 days) in the absence of virus. Thus, administration of a $10 \mu\text{g}$ dose on day 0 would result on day 7-8 in a level of HP bound to RBCs equivalent to $1.25 \mu\text{g}$ and should be protective against WNV challenge. Thus HPs developed for human use can be administered prophylactically to people at risk including healthcare workers responding to a biowarfare attack, soldiers on the battlefield, and travelers/soldiers visiting an endemic area.

In the SA model described in this report, animals protected against a lethal challenge by HP developed an immune response that left them protected to a secondary challenge with the same or alternative SA strain or upon re-challenge with *S. epidermidis*. These antibodies are clearly distinct from

the Spa27 MAb present in the HP since this MAb does not offer protection against a primary SA challenge and pharmacokinetic studies have demonstrated that the HP has a half-life of ~2.5 days and would not be present at day 28 during the rechallenge. If this is the case for protection from other pathogens, then HP treatment becomes a method for vaccinating an individual upon exposure to the pathogen. Thus, a soldier treated with an HP and then exposed to the target biothreat agent could gain protection from repeated exposure without further HP administration.

The mechanism by which an immune response was generated in HP protected animals is under investigation. One mechanism of achieving protective immunity is that the HP clears most, but not all of the pathogen and this reduced level would not be pathogenic, but sufficient to elicit an immune response. This mechanism has been suggested in studies utilizing a neutralizing antibody to a flavivirus (Tick Borne Encephalitis virus).¹⁴ In these studies, the antibody protected mice against a primary challenge and the surviving mice were resistant to homologous re-challenge due to antibody response against nonstructural viral proteins.¹⁴ An alternative mechanism is that HPs direct presentation of a pathogen to the immune system, possibly by transfer of the pathogen to macrophages or to dendritic cells, which are known to express CR1 on their surface.⁸ To distinguish between these two possibilities, reduction of pathogen load and immune presentation versus direct immune presentation, experiments will be conducted in which mice are protected from pathogen challenge by a neutralizing antibody or HP and development of a protective immune response monitored. These studies will take advantage of MAb 4G2 which can protect mice from a lethal WNV challenge when administered at a 10-fold higher concentration than those used in the WNV challenge described in this report (Figure 8).

HPs have the added advantage of immobilizing pathogens onto RBCs leading to their destruction by fixed tissue macrophages in the liver and spleen. Unlike neutralizing MAbs, HPs do not require complement fixation and or neutrophils for pathogen inactivation and destruction, therefore HPs would be effective therapeutics in individuals that are immunocompromised or have defects in complement including individuals that are on immunosuppressive drugs for transplants, chemotherapy for cancer, have HIV or have disease that effects complement activation.

The safety of HPs was demonstrated in studies using an HP derived by linking a CR1 MAb to a piece of double-stranded (ds) salmon sperm DNA (antigen heteropolymer, AHP) and testing in Phase I clinical trials in healthy humans and in systemic lupus erythematosus (SLE) patients.¹⁵ There were no safety issues seen in either volunteer population upon AHP administration and in SLE patients, AHP administration reduced the level of circulating anti-dsDNA antibodies (a hallmark of this disease) by binding to RBC. These trials demonstrated that HPs can be safely administered in humans and confirm the efficacy seen in animal models.

Taken together, the data from these studies and others demonstrate that HPs are a unique class of therapeutic that is amenable to provide treatment for multiple category A bioterrorism agents as well as common pathogens such as SA. HPs can be utilized both prophylactically for protection of people going into at risk areas, as well as, treatment of individuals post exposure. These studies also suggest that treatment of infection with HPs will provide protection against a secondary challenge to the same or similar pathogen. A major advantage of HPs over conventional MAb therapy is that non-neutralizing antibodies can be used to generate effective HP therapeutics that can provide protection/treatment for immunocompromised individuals. In addition, the ability to utilize non-neutralizing MAbs will provide for relatively easy and rapid development of HP therapeutics against new targets including other category A bioterrorism agents.

CONCLUSIONS

Bacterial and viral pathogens are serious threats as bioterrorism agents and considerable effort has been put forth to develop new vaccines and therapeutics for these agents. While vaccines are effective they do have limitations in that (1) protective immunity elicited by vaccination takes time; i.e. vaccines usually have to be administered more than once to achieve protection and maximum effect is usually not seen till several weeks after each administration, (2) vaccines cannot usually be used therapeutically since the time required to achieve a protective immune response will be longer than the first appearance of pathogenicity by a virus or bacterial infection and (3) vaccination of immunocompromised individuals has associated risks. The primate immune system has evolved to clear pathogens from the blood. When the immune system creates antibodies to a pathogen some of these antibodies will bind serum complement proteins to the pathogen. These immune complexes can bind to a privileged site on red blood cells (the complement receptor 1 protein). The immune complexes are then transported to the liver and spleen where they are removed from the surface of the red blood cell and destroyed, returning the red blood cell unharmed to the circulation.

Heteropolymers are a new platform technology that can enhance the ability of the primate immune system to remove and destroy pathogens. By linking together an antibody to a pathogen with an antibody to CR1, the heteropolymer can remove the pathogen from the circulation and deliver it for destruction without the requirement for generation of an antibody response to the pathogen. This “instant immunity” has been shown in a mouse model employing human CR1 on mouse red blood cells, to be an effective prophylactic against a lethal challenge with bacteria (*Staphylococcus aureus*) or virus (Vaccinia, West Nile). In these models efficacy was obtained with HPs that contained antibodies that did not neutralize the pathogen. Initial results suggest that heteropolymers can be used therapeutically against a bacterial infection. These results support the development of heteropolymers against bacterial (plague, tularemia) and viral (Smallpox, Ebola, Dengue) bioterrorism threats.

ACKNOWLEDGEMENTS

This work was supported by NIH grant 5 U01 AI054694-02 and Elusys Therapeutics Inc. Monoclonal antibodies to vaccinia were obtained from the US Army Medical Research Institute of Infectious Diseases (USAMRIID) under a Cooperative Research and Development Agreement (CRDA), Number DAMD17-01-006).

The authors would like to thank Dr. Mark Buller (St. Louis University Health Science Center) for help with testing vaccinia HPs. The authors would also like to thank Drs. Peter Mason and Nigel Bourne (University of Texas Medical Branch) for conducting testing of HPs with West Nile virus. The authors also want to thank Dr. David Perlin (Public Health Research Institute, Newark, NJ) for useful discussions in the course of this work.

REFERENCES

1. Nelson R. A. 1953. The Immune-Adherence Phenomenon. *Science*. 118, 733-737.
2. Nardin A., M. A. Lindorfer and R. P. Taylor. 1999. How are immune complexes bound to the primate erythrocyte complement receptor transferred to acceptor phagocytic cells? *Molecular Immunology*. 36: 827-835.
3. Schifferli, J. A. and R. P. Taylor. 1989. Physiological and pathological aspects of circulating immune complexes. *Kidney Int.* 35: 993-1003.
4. Reinagel M. L., M. Gezen, P. J. Ferguson, S. Kuhn, E. N. Martin, R. P. Taylor. 1997. The Primate

- Erythrocyte Complement Receptor (CR1) as a Privileged Site: Binding of Immunoglobulin G to Erythrocyte CR1 Does Not Target Erythrocyte for Phagocytosis. *Blood*. 89:1068-1077.
5. Lindorfer, M.A., Hahn, C.S., Foley, P.L., and Taylor R.P. Heteropolymer-mediated clearance of immune complexes via erythrocyte CR1: mechanisms and applications. *Immunol Rev*. 2001;183:10-24.
 6. Repik A., Pincus S. E., Ghiran I., Nicholson-Weller A., Asher D. R., Cerny A., Casey L. S., Jones S.M., Jones S. N., Mohamed N., Klickstein L., Spitalny G. L., Finberg R. W. 2004. A transgenic mouse model for studying the clearance of blood-borne pathogens via human complement receptor 1 (CR1). *Clinical and Experimental Immunology*, In Press.
 7. Lowy F. D. 1998. *Staphylococcus aureus* Infections. *N Engl. J. Med*. 339: 520-532.
 8. Nickells M, Hauhart R, Krych M, Bala Subramanian V, Geoghegan-Barek k, Marsh Jr. HC Atkinson JP. 1998. Mapping epitopes for 20 monoclonal antibodies to CR1. *Clin Exp Immunol*. 112: 27-33.
 9. Gentry MK, Henchal EA, McCown JM, Brandt WE, Dalrymple JM. 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am J Trop Med Hyg*.3:548-55.
 10. Hollinshead, M, Vanderplasschen, A, Smith, GL, Vaux, DJ. 1999. Vaccinia virus intracellular mature virions contain only one lipid membrane. *J Virol*; 73:1503-1517.
 11. Blasco, R, Moss, B., 1991.Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of a gene encoding the 37000-Dalton outer envelope protein. *J Virol*; 65:5910-5920.
 12. Payne, LG. 1980. Significance of extracellular enveloped virus in the *in vitro* and *in vivo* dissemination of vaccinia. *J Gen Virol*; 50: 89-100.
 13. Law, M, Hollinshead, R, Smith, GL, 2002. Antibody-sensitive and antibody-resistant cell-to-cell spread by vaccinia virus: role of the A33R protein in antibody-resistant spread. *J Gen Virol*; 83: 209-222.
 14. Kreil, TR, Maier, E, Fraiss, S, Eibl, MM. 1998. Neutralizing antibodies protect against lethal flavivirus challenge but allow for the development of active humoral immunity to a nonstructural protein. *J. Virol*. 72: 3076-3081.
 15. Iking-Konert, S., Stocks, S., Weinsberg, F., Engelbrecht, R Bleck, E., Perniok A., Fischer-Betz, R., Pincus, S., Nardone, L., Schneider, M. (2004) First clinical trials of a new heteropolymer technology agent in normal healthy volunteers and patients with Systemic Lupus Erythematosus: safety and proof-of-principle of the antigen-heteropolymer ETI-104 *Annals of the Rheumatic Diseases*. 63: 1104-1112.